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Antiinflammatory and antioxidant activities of different hemostatics and pulp capping materials applied on rat teeth

Biochemical effects of direct pulp capping with different hemostatics

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Abstract
Aim: This study aimed to investigate the anti-inflammatory and antioxidant activities of different hemostatic agents and pulp capping materials.
Material and Methods: Ninety-six Wistar albino male rats were divided into the following groups: Sterile Saline (SS), Sodium Hypochlorite (NaOCl), Mecsina Hemostopper (MHS). Occlusal cavities were prepared in the first molar teeth. Hemostatic agents were used to control bleeding and different pulp capping materials were applied on the exposed pulp area. Subgroups were created according to pulp capping materials (Dycal, Biodentine Theracal, MTA RepairHP). Half of the groups were left to wait for sacrifice on the 7th day and the others on the 28th day. Cardiac blood was taken to determine of antiinflammatory and antioxidant serum markers.
Results: The use of different hemostatic agents in terms of antioxidant (CAT, GPX) and anti-inflammatory (IL1-β, TNF-α, IL-6) activity was statistically significant ($p < 0.05$). There was no statistical significance between groups for SOD ($p > 0.05$). IL-6 was significantly higher on the 7th day than on the 28th day ($p < 0.05$). The anti-inflammatory activity reduced from the 7th to the 28th day, in contrast, antioxidant activity induced.
Discussion: The inflammation process was related to the hemostatic agents and the capping materials used in DPC treatment. MHS may be an alternative for DPC treatment for bleeding control.

Keywords
Anti-Inflammatory Effect; Antioxidants; Dentistry; Direct Pulp Capping; Mecsina Hemostopper

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Introduction

Direct pulp capping (DPC) is a treatment method involving the application of dental materials on the exposed pulp tissue for the survival of the pulp and the formation of reparative dentin. Cytokines are important markers in the repair process, in which immune cells come into play along with hard tissue formation. This process results in either a pulp repair with or without the dentine bridge, or in fibrosis or necrosis [1]. For this reason, the success of DPC treatment is still controversial for clinicians due to its unpredictable results [2].

Cytokines, which are released after exposure to pulp tissue, are polypeptide products that act as mediators of the immune response. IL-1β, which induces acute and chronic inflammation, is a cytokine that activates the natural and acquired immune system IL-6, an important cytokine in the formation of an acute phase response to tissue damage, works synergistically with IL-1β and TNF-α, which is known to have a significant effect in acute inflammation [3]. It has been reported that titers of IL-1β and TNF-α significantly increase in irreversible damage of pulpal tissue [4].

Antioxidant enzymes (Superoxide dismutase, Catalase, Glutathione Peroxidase) act as protection against oxidative damage that is caused by free radicals released during inflammation (such as H2O2). Superoxide dismutase (SOD) is the strongest antioxidant and the first detoxification enzyme [5]. Catalase, known as H2O2 oxidoreductase, is an antioxidant enzyme necessary for the the proper functioning of the organism [6]. Glutathione peroxidase, an intracellular antioxidant enzyme, plays a vital role in protecting the organism against free radicals [5]. Antioxidants can be natural and synthetic. Plant extracts contain some components with antioxidant properties [7].

Pulpal bleeding control is an important step in DPC treatment. For this purpose, sterile saline (SS), sodium hypochlorite (NaOCl) are used in traditional applications. SS, which can cause pulpal inflammation as a result of reactivation of hemorrhage and causing dislocation of the clot, has been shown as the material with the least cytotoxicity in the studies conducted [8]. NaOCl has been shown to have negative effects on stem cell survival and differentiation as a result of clinical studies and research [9]. It is also known to increase acute inflammation of vital cells [9.10]. Mecsina hemostopper (MHS) is a herbal extract made from herbal agents such as Glycyrrhiza glabra extract, Alpinia officinarum, Thymus serpyllum, Syzygium aromaticum, Hypericum perforatum, Vitis vinifera, Urtica angustifolia, Mentha arvensis. This leads to an erythrocyte aggregation by creating a protein network in the bleeding area and enables bleeding control [11].

Research was carried out to investigate and use new materials to predict and achieve desired success in dental practice [12]. Bioactive endodontic materials (BEM) should prevent pulpal inflammation and provide tissue healing. Calcium hydroxide, which is used as the gold standard in DPC treatment, has properties such as adhesion deficiency, deterioration after acid etching, dissolution against oral fluids [13]. Theracal resin-based material, which leads to repair dentine formation by increasing the pH on the pulp surface, and polymerizes with light, may cause pulpal inflammation due to its cytotoxic feature [14]. The issue of how MTA Repair HP, a new bioactive material, acts in the pulpal healing process has not yet been adequately clarified.

Due to the fact that the pulp tissue, which has a low amount of antioxidant activity, can give an inadequate recovery response to any injury, in this study, it was planned to investigate the anti-inflammatory and antioxidant response, MHS, which is provided with hemostasis and different bioactive materials, which is a new hemostatic agent with an herbal content. The first null hypothesis of this study is that different hemostatic agents will not affect anti-inflammatory markers on the 7th and 28th days. The second null hypothesis of this study is that the effectiveness of hemostatic agents will not differ in terms of antioxidant activity at different times.

Material and Methods

For the study, approval was obtained from the Ethics Committee of Sivas Cumhuriyet University (ID: 65202830-050.04.04-179). Wistar albino male rats, 4-6 months old, with a total weight of 200-250 g were divided into 3 main groups in order to carry out the application procedure of anti-bleeding agents. All experiments were conducted in compliance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals. The subjects were kept in standard test cages at 22-24 ° C, 55-70% humidity, 1 atm. pressure for 12 hours in a light/dark room and their health status was checked. The animals were anesthetized with intramuscular injection of ketamine HCl (25 mg/kg) and xylazine (10 mg/kg). Maxillary first molar teeth were cleaned with a small brush with %5 sodium hypochlorite and %0.1 chlorhexidine gluconate before cavity preparation. Class I cavities opened with dental burs under water cooling, approximately 1 mm perforation areas were created with a sterile probe tip. In Group SS, 0.09% Sterile Saline (Osel Drug Company), in Group NaOCl, 5% Sodium Hypochlorite Imicryl, Konya, Turkey and in Group MHS, Mecsina Hemostopper [Gesmir Company, Ankara, Turkey] were applied with sterile cotton pellets. The application times of the agents for hemostasis were determined as 5 minutes, 20 seconds and 20 seconds, respectively. Then the main groups were divided into 4 different subgroups according to the DPC application procedure (n = 8), and then, perforation areas were filled in with Calcium Hydroxide (C) [Dycal Dentsply, Caulk Milford, DE, USA]; Biodentine (B) [Septodont, Saint Maur des Faussés, France], Theracal (Th) [Bisco Inc, Schamburg, IL, USA] and MTA Repair Hp (MTA) [Angelus, Londrina, PR, Brazil] prepared in line with the manufacturing firm's instructions and the cavities were filled with glass ionomer cement (GC Fuji IXGP EXTRA, GC Corporation, Tokyo, Japan) to prevent microleakage.

Half of the rats were sacrificed on the 7th day and the rest on the 28th day. Cardiac blood was collected under anesthesia. It was centrifuged in a cooled centrifuge (Hermle Z326) to obtain serum from the collected blood. Enzyme-linked immunosorbent assay (ELISA) test was performed to compare antioxidant enzymes (Catalase, Glutathione Peroxidase, Superoxide Dismutase) and anti-inflammatory (IL-1β, IL-6, TNF-α) activities.

ELISA Test Procedure Blood serums were obtained after centrifugation and collected in Eppendorf tubes. IL-1β, IL-6, TNF-α, Cat, GPX, SOD were analyzed spectroscopically using an ELISA device (Thermo Multiskan) according to kit procedures

using the Bioassay Technology Laboratory commercial test kits. The respective reagent and antibody were added to each well. Streptavidin-HRP (50µl) was added to each standard and sample. After incubation, the wells were washed 5 times at 37 °C and 60 minutes in the dark environment. Finally, the absorbance of the standard and samples was measured in the ELISA device at 450 nm wavelength.

Statistical analysis was performed in the SPSS 22.0 program used to evaluate the data, and the significance value was taken as p <0.05. The normality of data distributions was analyzed with the Kolmogorov-Smirnov test. The Kruskal-Wallis for nonparametric testing and One-way ANOVA for parametric testing were used for variables to compare the studied parameters for the statistical difference. Differences were considered significant at p <0.05.

Results

When the serum levels of rats sacrificed on the 7th day were evaluated, after the normality tests were applied, non-parametric tests were conducted since the Cat, SOD, IL1β and IL-6 parameters did not show normal distribution. The Kruskal-Wallis test was used to compare these values. Since GPX and TNF-α parameters had normal distribution, One-Way ANOVA test was applied (Table 1).

When comparing Cat, GPX, IL1-β, TNF-α, IL-6 values between groups using different haemostatic agents in terms of antioxidant and anti-inflammatory efficacy, a statistically significant difference was obtained (p<0.05). There was no statistical difference between the groups in terms of SOD enzymes (p>0.05). Cat value was found to be higher in hemostasis with MHS coated with Biodentine and MTA Repair Hp groups than hemostasis SS capped by MTA Repair Hp groups. Average GPX parameter for MHS-Cal, SS-MTA, NaOCl-B groups is higher than for the other groups. The mean IL-1β cytokine is lower in the SS-MTA group than the MHS-B, MHS-MTA, SS-B, NaOCl-C and NaOCl-The averages.

The mean value of the IL-1β cytokine of the SS-Cal group is lower than those of the NaOCl-C, NaOCl-The groups. The average TNF-α enzyme is higher in the groups MHS-The, SS-MTA, SS-C, NaOCl (C, B, MTA) than in the others. The mean of IL-6 was higher in the groups that were covered with Theracal and MTA material by ensuring hemostasis with NaOCl.

When the serum markers of the rats sacrificed on the 28th day were evaluated, Cat, SOD, IL1-β and IL-6 parameters did not show normality, although the Kruskal-Wallis test was applied. One-way ANOVA test was applied because GPX and TNF-α parameters, belonging to the same group, showed normality (Table 2).

The average SS-MTA Cat parameter was lower than other groups. NaOCl-The Cat parameter average was higher than in the other groups. SOD averages did not show statistical differences according to the applied materials (p> 0.05).

When blood serum markers were evaluated on the 7th and 28th day, the mean IL-6 parameter was significantly higher on the 7th day than on the 28th day (p > 0.05) (Table 3).

Cat, GPX, IL-1β and SOD levels in the MHS group were not statistically significant on the 7th day than on the 28th day (p> 0.05). However, the difference between TNF-α and IL-6 levels was found to be statistically significant (p >0.05).

When comparing the variability of blood serum markers on the7th and 28th days of different materials used in the study, there were statistical differences in IL-6 and TNF-α levels (p<0.05). IL-6 levels comparison between days 7 and 28 was statistically significant between 7th and 28th day (p<0.05). The levels of IL-6 and Cat showed a statistically significant difference on the 7th and 28th days (p<0.05).

Table 1. Antioxidant and antiinflammatory serum markers on the 7th day

	ANTIOXIDANT MARKERS			ANTIINLAMMATORY MARKERS		
	Catalase Mean ±sd	GPX Mean ±sd	SOD Mean ±sd	IL1β Mean±sd	TNF -α Mean±sd	IL6 Mean±sd
Mecsina						
Calcium hydroxide	3,988 ±1,63	79,966 ±14,66	0,033 ±0,00	7,566 ±1,18	40,979 ±9,85	0,519 ±,07
Biodentine	6,393 ±1,56	63,851 ±9,87	0,027 ±0,00	11,493 ±3,98	31,368 ±0,95	0,536 ±0,05
Theracal	2,882 ±1,43	76,211 ±3,72	0,037 ±0,00	5,145 ±0,25	47,471 ±1,40	0,459 ±0,08
MTA Repair Hp	6,431 ±0,51	60,751 ±4,30	0,031 ±0,00	9,306 ±1,08	30,721 ±1,63	0,568 ±,09
Sterile Saline						
Calcium hydroxide	3,357 ±0,64	73,398 ±4,81	0,035 ±0,00	5,695 ±0,79	42,306 ±3,04	0,642 ±0,04
Biodentine	3,079 ±0,83	68,641 ±1,00	0,032 ±0,00	11,001 ±2,20	36,338 ±6,94	0,733 ±0,14
Theracal	2,006 ±,0,62	69,586 ±2,30	0,121 ±0,16	6,587 ±0,21	34,683 ±1,42	0,519 ±0,10
MTA Repair Hp	6,207 ±2,79	84,698 ±7,16	0,041 ±0,00	4,010 ±0,61	50,009 ±3,20	0,532 ±0,09
Sodium Hypochlorite						
Calcium hydroxide	2,983 ±0,38	75,258 ±5,97	0,034 ±0,00	11,004 ±1,01	44,995 ±2,33	0,537 ±0,07
Biodentine	2,973 ±0,37	82,161 ±7,53	0,045 ±0,00	4,053 ±0,08	57,904 ±2,11	0,605 ±,07
Theracal	4,849±3,26	58,542 ±7,53	0,034 ±0,00	11,226 ±0,90	35,130 ±5,02	1,143±0,30
MTA Repair Hp	3,033 ±1,35	69,328 ±4,43	0,035 ±0,00	8,180 ± 1,89	42,975 ±2,17	0,955 ±,15
Test	KW=23,758	F=5,549	KW=17,636	KW=40,173	F=14,878	KW=30,673
p	0,014*	0,000*	0,090	0,000*	0,000*	0,000*

*p<0,05

Table 2. The values of antioxidant and antiinflammatory serume according to different hemostatic agents on the 28th day.

	ANTIOXIDANT MARKERS			ANTIINLAMMATORY MARKERS		
	Catalase Mean ±sd	GPX Mean ±sd	SOD Mean ±sd	IL1β Mean±sd	TNF -α Mean±sd	IL6 Mean±sd
Sterile Saline						
Calcium hydroxide	5,546±1,94	93,815 ±14,18	0,036±0,00	6,373±0,14	46,329±10,89	0,299 ±0,07
Biodentine	4,121±1,63	56,790 ±7,87	0,114 ±0,16	8,604±2,16	36,728 ±4,43	0,527 ±0,07
Theracal	6,047±2,38	77,758 ±4,84	0,031 ±0,00	8,567±,63	42,881 ±1,33	0,377 ±0,02
MTA Repair Hp	1,906±0,72	67,996 ±10,46	0,104 ±0,14	12,278±1,29	29,091 ±8,08	0,370 ±0,06
Sodium Hypochlorite						
Calcium hydroxide	4,993±1,24	93,676 ±0,75	0,037 ±0,00	6,251±1,26	56,069±1,26	0,324 ±0,04
Biodentine	4,403±0,56	63,730 ±4,59	0,034 ±0,00	9,141±1,75	42,219±3,83	1,129 ±0,63
Theracal	9,176±0,54	41,145 ±3,13	0,035 ±0,00	10,817±2,54	23,323±2,11	0,344 ±0,02
MTA Repair Hp	3,007±0,74	67,812 ±8,22	0,034 ±0,00	5,502±0,51	46,784±6,78	0,648 ±0,06
Mecsina						
Calcium hydroxide	2,993±1,61	84,800 ±21,09	0,036 ±0,00	7,860±3,24	48,178±10,83	0,439 ±0,05
Biodentine	6,075±2,86	58,637 ±15,01	0,034 ±0,00	8,644±3,65	41,149±12,09	0,381 ±0,05
Theracal	3,251±1,49	70,303 ±13,74	0,036 ±0,00	6,558±1,25	42,499±5,01	0,390 ±0,05
MTA Repair Hp	4,756±0,90	74,411 ±11,14	0,035 ±0,00	5,177±0,34	47,397±3,68	0,630 ±0,15
Test	KW=28,212	F=7,674	KW=5,827	KW=29,268	F=6,466	KW=34,589
p	0,011*	0,000*	0,885	0,002*	0,000*	0,000*

*p<0,05

Table 3. Difference between antioxidant and anti-inflammatory serum markers between 7th and 28th days.

	n	Mean±sd	Test	p
Catalase				
7th day	48	4,01 ±2,04	z=-1,451	0,147
28th day	48	4,69 ±2,30		
GPX				
7th day	48	71,86 ±10,11	t=0,325	0,746
28th day	48	70,90 ±17,76		
SOD				
7th day	48	0,04 ±0,04	z=-0,616	0,538
28th day	48	0,04 ±0,06		
IL1B				
7th day	48	7,93 ±3,08	z=-0,333	0,739
28th day	48	8,03 ±2,70		
TNF-α				
7th day	48	41,24 ±8,67	t=-0,330	0,742
28th day	48	41,88 ±10,44		
IL-6				
7th day	48	0,64 ±0,22	z=-4,917	0,000**
28th day	48	0,48 ±0,28		

*p<0.05, **p<0.01

Discussion

This study aimed to investigate the anti-inflammatory and antioxidant activities of different hemostatic agents and capping materials in the early and late periods. The first null hypothesis, which states that the data obtained from the study did not have any effect on different days of inflammatory markers, was rejected. The response of pulp tissue to different hemostatic agents and capping materials was statistically significant at different times and the difference in the inflammatory response to MHS plant extract and other haemostatics was statistically significant. The 2nd null hypothesis, which states that different haemostatics did not affect antioxidant markers on days 7 and 28, was also rejected. Antioxidant markers showed a difference with responses given to different hemostatic agents on days 7 and 28.

Many experimental animals are used in the creation of pulpal injury in the literature. In the current study, rats were used because of the similarity of the oral microflora and the fact that pulp and periodontal tissues are similar to to those of humans [15]. An inflammatory response and inflammatory cells similar to that of human are seen in rat pulp tissue [15,16].

The expected pulpal healing process following the application of DPC materials on damaged pulp tissue depends on the response of the inflammatory process to the material. This process is related to the cytotoxicity of these materials used in capping. The resulting inflammatory response is also related to the induction of cytokine production of capping materials. Cytokines used in studies in order to understand the mechanisms of pulpal diseases in the literature involve the investigation levels of IL-1, IL-6 and TNF-α [17,18]. In this study, the in vivo method was preferred because observing the clinical success of the DPC procedure is difficult with in-vitro. The ELISA test is considered one of the most reliable methods to detect cytokine antigen [19].

SS, formed as a control group, is the most commonly used hemostatic agent in the clinic, although the success and effectiveness of pulpal treatments are limited [8]. It is observed that the inflammatory response is generally higher in groups that have hemostasis with SS.

NaOCl has a disadvantage due to its cytotoxicity to pulp cells, although it is recommended as an alternative hemostatic agent due to its bacterial activity [20]. However, it is emphasized that its use in concentrations between 2% and 5% is not cytotoxic to pulp cells [21]. For this reason, it was observed that

the 5% concentration of NaOCl inflammation was preferred and increased the release of cytokines in the acute period. Inflammatory reaction starts immediately in a healthy pulp tissue as a result of pulpal injury [22].

In the MHS group, which is completely obtained from plant extract, it was observed that while TNF-α levels on the 28th day were higher compared the 7th day ; on the contrary, IL-6 levels appear to decrease. It is thought that this may be due to the fact that IL-6 is more pronounced in acute inflammation or that the anti-inflammatory activities of the hemostatic agent used are different.

IL-1, IL-6 and TNF-α are cytokines that occur in the management of pulpal inflammation. TNF-α values, which are an important mediator in the creation of host response in the early period of injury, have lower averages in the groups that achieved haemostasis with MHS. Acute inflammation turns into chronic inflammation, which is characterized by the production of more diverse pro-inflammatory cytokines and antioxidant activity over time. In oral diseases, IL-6 is a cytokine involved in trauma, infection response and inflammation, and can be found at high levels in isolated from blood serum when irreversible pulpitis is seen compared to healthy pulp tissue [23]. The lower levels of IL-6 levels in groups with hemorrhage with MHS compared to the hemostatic used on day 7 is an important finding in terms of the fastest control of the anti-inflammatory response.

However, the statistically insignificant increases observed in some groups suggest that this may be due to the different chemical properties of the capping materials. When the groups in this study are evaluated, there was a general decrease in IL-6 levels on day 28th. This indicates that pulpal inflammation slowed down in the following days. When these findings are examined in terms of the response of the organism on different days, a decrease of IL-6 levels while an increase in the value of IL-1β and TNF-α, which show synergistic effect, corresponds to the literature [4]. This finding shows that the cellular response given in the following period changed from an acute phase to a chronic phase.

It is possible that the capping materials modulate the inflammatory response. Cavalcanti et al. examined cytokine release in their study using a healthy human donor and found a high level of IL1-β release in the MTA group [23]. The probable reason for our results that do not match with the data of their study can be explained by the fact that the MTA Repair Hp material is different from the conventional MTA; it is treated with a hemostatic agent before the capping materials are applied, and the experimental procedure performed in the rat pulp is different. In vitro studies show that non-resin containing Biodentine and MTA material increase proinflammatory cytokine secretion compared to the resin-containing Theracal material [10,14,23]. In 2017, Jeanneau et al. 's study on immunofluorescence reported that Biodentine material reduced proinflammatory cytokine (IL-8) released from pulpal cells, but this release was high in the resin-containing Theracal material [14]. It is thought that the cytokine studied in the forementioned study is different from this study and the interaction of fibroblasts obtained from human pulp with the capping materials is not parallel with the data of this study. These data confirm that inflammation is suppressed on the 28th day.

Antioxidants have been used for many purposes (pulp capping, dentin hypersensitivity, remineralization, bond strength after bleaching) in the field of dentistry [5,11]. The response to pulpal damage is a rather complex process. Antioxidant enzymes are present in the healthy dental pulp [16,23]. In their study, Tulunoğlu et al. examined SOD levels in healthy and pilpulistic pulp tissues and found difference between these two tissues in terms of enzyme activity [24]. In the current study, it was observed that SOD levels generally increased, but in some groups, there were statistically insignificant decreases. This variability can be expressed as the difference in components of the capping materials used in combination with hemostatic agents. There are study findings showing that SOD enzyme activity increases with inflammation [19,23,24]. Supporting the literature, there was an increase in the amount of SOD on the 7th and 28th days. Decreased SOD activity in the MHS group can be explained as suppression of inflammation. The literature has reported effectiveness against free radicals formed after pulp capping antioxidants such as N-acetylcysteine Ankaferd hemostopper, propolis, and catalase applied as local antioxidants, have been reported to delay pulpal inflammation [6]. The marked decrease in IL-6 levels on the 28th day was that the acute infection response of IL-6 cytokine was stronger and decreased in the following period; it can also be explained by the effectiveness of the shot at antioxidant enzyme levels although it has no statistically any differences.

The clinical study in healthy humans, with reversible and irreversible pulpitis has revealed that catalase activity is an important defense against free radicals and is statistically significant [25]. It is not surprising that the results of this clinical study carried out by Esposito et al. do not correspond with the results of the current study. It is thought that differences in experimental models, materials, and treatment methods used are reflected in the results. Within the limitations of the present study, it was concluded that the course of inflammation was related to the hemostatic and the coating material used on the 7th day and 28th day. The results indicate that the changes in the rate of inflammatory cytokines on the 7th day and on the 28th day are important for the control of inflammation and that early inflammation is controlled by MHS in an early period, and can be used as an alternative to SS and NaOCl in clinical practice. However, more research is needed to observe the results that may occur with clinical use.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
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